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PRINCIPAL INVESTIGATOR: Victor Arvanian

CONTRACTING ORGANIZATION: Research Corporation of Long Island, Inc.
                                 Northport, NY 11766

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14. ABSTRACT
During Year 2 we have used anti-NG2-antibodyes made in Dr. Levine's lab and accomplished following studies. 1) Experiments described in Specific Aim (SA)2: we demonstrate that a single intraspinal injection of anti-NG2-antibody (Ab) removes conduction block and restores transmission deficits induced by acute NG2. 2) Experiments described in SA3 and SA4 related to chronic effects of NG2-Ab delivered via osmotic minipump: we demonstrate that prolonged infusion of anti-NG2-Ab strengthens transmission, promotes anatomical plasticity (SA3) and improves locomotor function and coordination in automated Catwalk gait (SA4) after chronic hemisection SCI. 3) We have succeeded to create the recombinant single chain (scFv) antibody and confirmed expression of anti-NG2-Ab; at present, AAV10 encoding the anti-NG2 scFv is being constructed in PENN vector core. During year 3 we will examine functional effects of this AAV-NG2-Ab construct, and accomplish SA3 and 4. If successful, this AAV-NG2-Ab construct will be the novel effective and clinically relevant treatment to facilitate recovery after SCI. Four papers have been published during 2011-2012 period; two other papers have been submitted for publication.

15. SUBJECT TERMS Completed: (2) proved that acute injections of anti-NG2 antibodies (Ab) prevent block of axonal conduction induced by acute NG2. (3) chronic delivery of NG2-Ab via mini-pump improves axonal conduction, plasticity and recovery of function after chronic SCI. Successfully created scFv Ab; its functional effects will be examined at Year3 to complete Project.

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Introduction

Regeneration of fibers in damaged spinal cord is quite limited and strategies to strengthen surviving connections in partially injured spinal cord appear to be more feasible for improving recovery of function (Arvanian et al. 2006a; Alilain et al., 2011; Garcia-Alias et al. 2011; Schnell et al. 2011). After SCI the diminished ability of the spared fibers to transmit signals during chronic stage of contusion (Hains et al. 2004; Arvanian et al. 2006b; James et al. 2011), compression (Nashmi and Fehlings 2001; Ouyang et al. 2010) and lateral hemisection (HX) (Arvanian et al. 2009) injuries have been reported. We have developed a method of intra-axonal recordings from anesthetized adult rats. Using this method we found that transmission deficits in damaged spinal cord after chronic HX are the result of reduced conduction in uncut axons (Hunanyan et al. 2011). Since elevated level of chondroitin sulfate proteoglycans (CSPGs) in the vicinity of the injury has been reported to be a major obstacle for recovery after SCI (Snow et al., 1990; Jones et al., 2002), the general strategy of this proposal is: (i) to identify individual CSPGs that might be responsible for the conduction deficits in the surviving fibers, (ii) to design a specific treatment that will neutralize these CSPGs, and (iii) to determine whether neutralization of these factors will strengthen synaptic effects of surviving descending fibers and improve functional recovery in adult rats after HX injury. Keeping up with the schedule of our study, During Year1 we found that among CSPGs which level is up-regulated in the vicinity of SCI, NG2 is blocks axonal conduction, but other CSPGs do not. During reporting Year2 of the Project we have completed research tasks for Year 2 and examined effects of NG2-Antibody delivered acutely and via osmotic mini-pump, as described in our SOW. Some results of experiments supported by DOD Proposal has been reported at three scientific meetings and three articles have been published in scientific journals. One review article and one article describing our recent findings (below) have been submitted for publication.

Body

1. Specific Aim #1 of our project has been accomplished during Year 1. We have examined and compared acute effects of several individual CSPGs on axonal conduction and synaptic transmission in intact spinal cord. We found that among CSPGs whose levels are mostly elevated after spinal cord injuries (i.e. NG2, neurocan and aggrecan), NG2 induced a dramatic depression of axonal conduction and transmission to individual motoneurons in intact spinal cord, but neurocan or aggrecan did not alter axonal conduction.

2. Specific Aim #2 has been accomplished during reporting Year 2. We have examined acute effects of anti-NG2-antibody (Ab) (made in Dr. Levine lab). We demonstrate that acute intraspinal injections of NG2-Ab prevented an acute block of conduction induced by NG2 (Fig. 1).
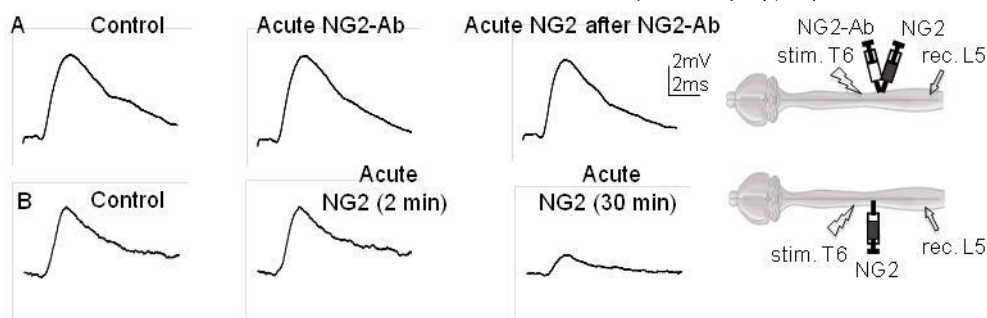

Figure 1. Intracellular recordings demonstrating acute effects of NG2 antibody on removing inhibitory effect of NG2 on axonal conduction. Representative traces of EPSPs recorded intracellularly from L5 motoneurons and evoked by stimulation of T6 ventrolateral funiculus in same non-injured adult rat, before and after of intraspinal injections of NG2-Ab or NG2. Diagrams show positions of stimulation (stim.) electrode in T6, recording (rec.) electrode in L5 and injection micropipette at T10. A, Superimposed averaged responses showing absence of inhibitory effect of NG2 on conduction in presence of NG2-Ab; control (before injection), 30min after injection of NG2-Ab and 30 minute after injection of NG2 which was injected 30 min after NG2-Ab injection respectively. B, Superimposed averaged responses recorded from the opposite side of the cord in same rat, showing depression of EPSPs after injection of NG2 in absence of NG2-Ab; control (before injection), 2 minutes and 30 minutes after injection of NG2 respectively.

In a previous study we found that intraspinal injections of purified NG2 in the vicinity of axons in lateral white matter of non-injured animals depressed transmission to L5 motoneurons through these axons (Hunanyan et al., 2010). In the current study, we examined the ability of the acute administration of NG2-Ab to prevent these inhibitory effects in the intact spinal cord.

All procedures were performed on adult, female Sprague-Dawley rats (~210 g) in compliance with Institutional Animal Care and Use Committee policies at SUNY-Stony Brook and the Northport VAMC.

Animals were deeply anesthetized using a ketamine/xylazine mixture (80 mg/kg/10 mg/kg) injected i.p. for induction and supplemented during experiment with 1/5 initial dose injected i.m. if needed. Expired CO_2 and heart rate were continuously monitored. An automatically controlled heating pad was used to maintain animal body temperature at 36.70C. Dorsal laminectomies were performed at T5-T7 (for placement of stimulation electrode rostral to HX) and L1-L6 (for placement of recording and stimulation electrodes at L1 caudal to HX). Intracellular responses (Axoprobe amplifier: Molecular Devices, Inc.) were recorded from L5 motoneurons and evoked by electric stimulation of ventrolateral funiculus (VLF) at T6 or L1 as described previously (Arvanian et al., 2009; Hunanyan et al., 2012). Briefly, motoneurons were impaled with sharp, glass microelectrodes (3M K-Acetate; 50-70 MΩ resistance) and identified by their antidromic response to stimulation of the cut L5 ventral root. In order to examine acute effects of NG2 antibody, we used intraspinal injection of NG2 and NG2-Ab during continuous recording from a single motoneuron. A custom-made two barrel pipette was fabricated by gluing together two separately pulled glass pipettes, 20 μm diameter each, at an angle of ~20o so that the tips were within 10 μm, of each other. One tip was filled with NG2-Ab and the other tip was filled with NG2 (Fig. 1). The tip of this two barrel pipette was positioned in the T10 lateral white matter of the spinal cord using a micromanipulator to ensure that the injections were made in the vicinity of axons that were being stimulated: the position and depth of the tip of the injecting pipette (at T10) were determined by adding 100 μm to the position and depth of the stimulating electrode (at T6) (to account for the greater thickness of the cord in T10).

After measuring the control intracellular responses evoked by electric stimulation of lateral white matter at T6, the NG2-Ab (1 μl) was slowly injected from one barrel and responses were measured again. 30 min following NG2-Ab injection, purified NG2 was injected from the other barrel and responses were measured again. We found that intraspinal injections of NG2-Ab did not induce changes in the amplitude of intracellularly recorded EPSPs, but prevented the block of EPSPs by subsequent NG2 injection. Amplitude of control intracellular responses was 5.6 ± 0.8 mV, after 30 min of Ab injections 5.7 ± 0.9 mV and after 30 min of NG2 injection (following Ab injection) 5.5 ± 0.6 mV ($p > 0.05$, n = 6 rats, 7-8 cells/rat). After completion of these injections and recordings on one side of the spinal cord, the stimulating and recording electrodes were removed and positioned symmetrically in the opposite side of the cord and the recordings were performed following injections of NG2 only. A micropipette containing NG2 was positioned between the stimulating and recording electrodes 5 mm caudal off the position of the initial injection at the opposite side of the cord. Under these conditions, intraspinal injections of NG2 induced a depression of the responses recorded intracellularly from L5 motoneurons. NG2 induced depression vs control responses was 72 ± 7% ($p < 0.05$, n = 6). Consistent with the effects of intraspinal injections of NG2 alone in a previous study (Hunanyan et al., 2010), intraspinal injections of NG2 did not induce immediate changes in the shape of EPSPs, but depressed EPSP amplitude 20-25 min post-injection.

These results show that the acute administration of NG2-Ab prevents the blockade of axonal conduction induced by acute NG2. Moreover, these results demonstrate that the approach of administering NG2-Ab via intraspinal injections blocked the acute inhibitory effects of NG2 on axonal conduction only in the vicinity of the intraspinal injections. This suggests the possibility that intraspinal injections of the NG2-Ab may be useful for local neutralization of NG2 in the areas where needed such as the site of SCI.

3. Specific Aims #3,4. A portion of SA#3 and SA#4 related to chronic administration of NG2-Ab via osmotic mini-pump after chronic SCI has been accomplished during Year2. During the past year we found that chronic delivery of NG2-Ab via osmotic mini-pump improves recovery of locomotor function (3a), synaptic transmission (3b) and anatomical plasticity (3c) after a lateral hemisection (HX) SCI.

Experimental design. Four groups of rats were used in chronic experiments for behavior, electrophysiology and anatomical tracing. Animals in the non-injured group received sham laminectomy and no treatments or injuries; HX only group received a hemisection injury at the T10 level and no treatments; NG2-Ab group received HX injury at T10 followed immediately by surgical implantation of an osmotic mini-pump to deliver an NG2-Ab mixture of two function-blocking antibodies 69 and 147; control-Ab group received HX injury at T10 and delivery of control NG2 non-neutralizing 132 antibody immediately after surgery via implanted osmotic mini-pumps. After SCI and pump implantation, animals were evaluated for locomotor recovery for 8 weeks after which electrophysiological or tracing experiments were performed. Production, derivation and specificity of two monoclonal NG2 function neutralizing antibodies, designated as 69 and 147 and control NG2 function non-neutralizing antibody 132 used in the current study have been done in laboratory of Dr. Levine as described previously (Ughrin et al., 2003; Tan et al., 2006).

Spinal cord injuries and treatment delivery. Animals were deeply anesthetized with 3% isoflurane in 100% O_2, then transferred to a face mask delivering 1.5 % isoflurane in 100% O_2 to maintain anesthesia. A water circulating heating pad was used to maintain body temperature during surgeries. Dorsal laminectomy was

performed to expose T10 spinal cord level. A tip of an iridectomy scissor was inserted through the midline from dorsal to ventral and the left hemicord was completely transected as previously described (Arvanian et al., 2009). Intrathecal administration of NG2 neutralizing antibody or control (non-neutralizing) antibody was given as previously described (Weinmann et al., 2006; Schnell et al., 2011). Briefly, a fine intrathecal catheter was inserted from lumbar L2 and pushed to T10 to deliver a total volume of 2mL (concentration: 500µg/ml for each antibody) of antibody from an osmotic minipump (Alzet 2ML2; 5µl/h, Cupertino, CA) over two weeks. The minipump, placed sub-cutaneously, was connected to the intrathecal catheter via tubing sutured to the back muscles for stabilization. The administration of antibodies began immediately following the injury with the delivery of 1µl of the corresponding antibody directly to the injured spinal cord. The muscles were sutured in layers and skin closed with surgical clips followed by subcutaneous injections of antibiotic (Baytril 5mg/kg), analgesic (Buprenorphine 0.01 mg/kg) and 5ml of sterile lactated Ringer`s solution. Multiple studies, using a similar delivery technique, have revealed excellent distribution and penetration of antibodies infused intrathecally throughout the spinal cord of adult rats and monkeys (Weinmann et al., 2006) and is currently used in clinical trials to deliver Nogo-A antibody to human spinal cord. The presence of infused NG2-Ab bound to cells expressing NG2 within the lesion penumbra in adult rat spinal cord has been previously confirmed (Tan et al., 2006). Reconstruction of the injury epicenter in all animals demonstrated that the mean lesion size was virtually identical for all groups (Fig. 5). Five animals with under-hemisection or over-hemisection were removed from study; other 4 animals excluded 2 days post-injury based on open field performance.

3a. *Behavioral assessment.*

For quantitative assessment of locomotor function we have used automated CatWalk gait analysis. Animals were pre-trained to cross a special glass runway where their footprints were captured by a high speed camcorder. Quantitative assessment of footprints and analyses were done using CatWalk XT software (Hamers et al., 2001) (CatWalk; Noldus Information Technology, The Netherlands) beginning at 2 weeks post-operation, i.e. time point when animals exhibited weight-supported stepping. Data from three complete runs for each animal were collected and gait parameters such as Stride Length and Base of Support, objective measurements of locomotor deficits after lateral HX (Garcia Alias et al., 2011), were compared between experimental groups.
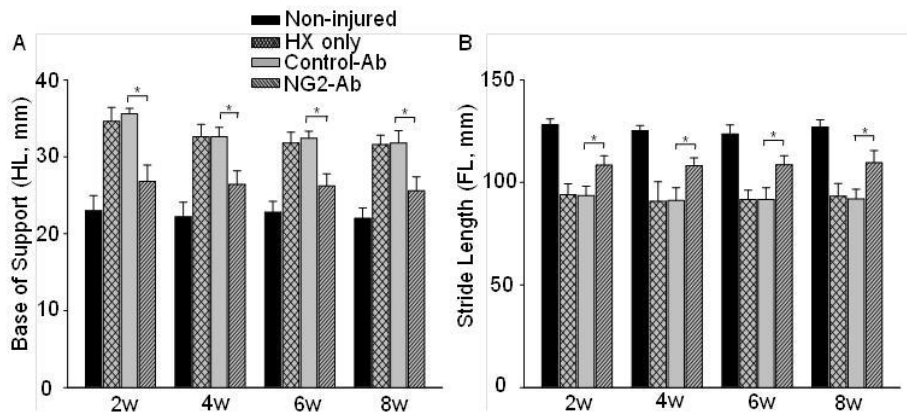

Figure 2. Effect of NG2-Ab treatment on locomotor function after chronic HX injury. A, CatWalk gait analysis to show that treatment with NG2-Ab improved base of support of hindlimbs. B, CatWalk gait analysis to show that NG2-Ab treated group showed significant improvement in stride length of forelimbs compared to control-Ab treated and HX-only groups. Note that results of CatWalk analysis demonstrate that beginning from two weeks throughout eight weeks post-injury (last time point of experiment) animals from NG2-Ab group showed significant improvement of function compared with control-Ab treated or HX only group. Note that control-Ab treated group didn't show any difference versus HX-only group on any test.

Gait parameters are considered to be a sensitive assessment of locomotor function in the case of HX SCI (Garcia-Alias et al., 2011). We therefore measured locomotor function using the Catwalk device starting from week 2 when weight support stepping of four paws was evident. All injured animals showed significant impairment compared to non-injured animals (Fig. 2). Control-Ab treated and HX non-treated animals showed same impairment and similar pattern of recovery in both base of support distance (HX-only: 31.6 ± 1.2 mm; HX/control-Ab: 31.8 ± 1.8 mm at 8 weeks post-injury; vs 22 ± 1.3 mm non-injured) and stride length (HX-only: 93.2 ± 6.2 mm; HX/control-Ab: 92 ± 4.7 mm at 8 weeks post-injury; vs 127 ± 3.4 mm non-injured). NG2-Ab treated animals showed significant improvement of function, evident by narrower base of support and an increase in stride length compared to control-Ab treated starting from week 2 and throughout the experiment

$(25.6 \pm 1.8$ vs 31.8 ± 1.5 mm, $p < 0.05$, Fig. 2A) for base of support and $(109.6 \pm 5.9$ vs 92 ± 4.7 mm, $p < 0.05$, Fig. 2B) for stride length at 8 weeks post-injury.

3b*. Chronic infusion of NG2-Ab improves synaptic plasticity in damaged spinal cord.*
We next examined whether chronic treatment with NG2-Ab improves transmission after a chronic HX SCI using a clinically relevant intrathecal delivery method. In this study NG2-Ab was delivered to the vicinity of HX lesion via osmotic minipump chronically for two weeks, which corresponds to the time point when accumulation of CSPGs around HX reaches its maximum level (Garcia-Alias et al., 2011). Rats received HX injury and immediately after the HX were implanted with a subdural catheter delivering either NG2-Ab or control-Ab from osmotic minipumps for two weeks. Eight weeks after surgery and behavioral testing, we recorded intracellularly from L5 motoneurons the responses evoked by electric stimulation of lateral white matter at T6 (i.e. rostral to T10 HX) and L1 (i.e. caudal to HX level) (Fig. 3).
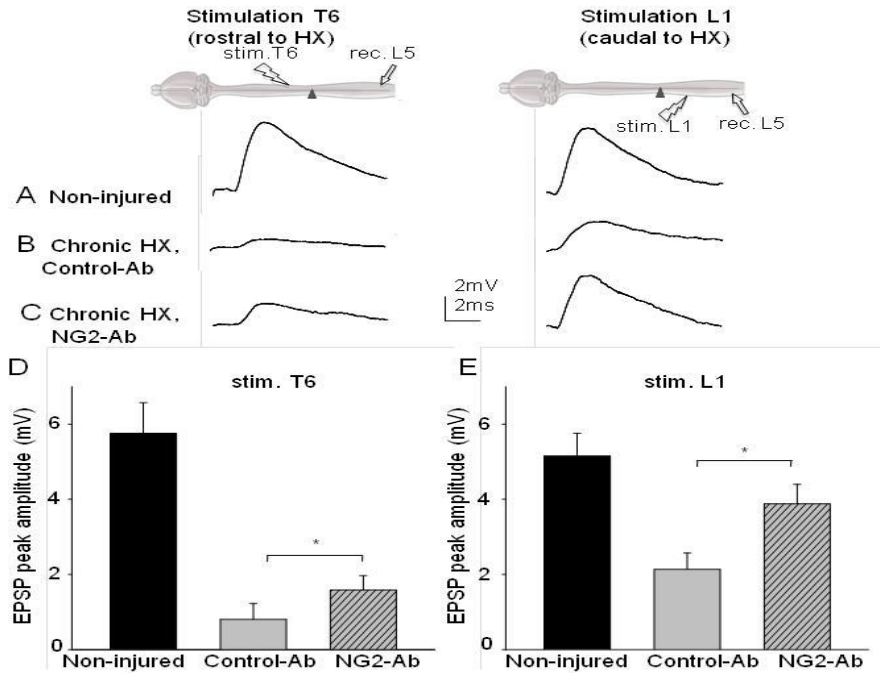

Figure 3. Intracellular recordings from L5 motoneurons demonstrating that chronic treatment with NG2-Ab partially restores transmission to L5 motoneurons. A, Superimposed averaged traces demonstrating monosynaptic responses recorded from L5 motoneuron and evoked by electric stimulation of T6 and L1 in same intact spinal cord, respectively. B, Superimposed averaged EPSP responses recorded from chronic HX rats treated with control-Ab. C, Superimposed averaged EPSP responses recorded from NG2-Ab treated animals showing larger amplitude of synaptic responses compared to control-Ab treated animal. (D, E) Summary of results demonstrating significant improvement of transmission to L5 motoneurons from T6 segment rostral to HX injury and L1 segments caudal to injury in NG2-Ab treated animals, respectively. Diagrams show positions of the recording electrode in L5 and stimulation electrodes at T6 and L1. Asterisks represent significant difference between corresponding graphs (p < 0.05).

We found that in animals that received HX injury and chronic treatment with a control-Ab, responses of L5 motoneurons evoked from both T6 and L1 levels were depressed significantly compared to non-injured animals. The mean amplitude of these diminished responses was 0.8 ± 0.5 mV from T6 and 2.1 ± 0.4 mV from L1 (Fig. 2, n = 5 rats, 37 cells total), i.e. similar to the amplitude of corresponding responses in animals that received a HX SCI and no treatment in previous study (~0.9 mV from T6 and ~2.5 mV from L1; Arvanian et al., 2009). In animals treated with NG2 function blocking antibody, the amplitude of the responses from T6 increased significantly $(1.58 \pm 0.38$ mV, n = 7, $p < 0.05$, Fig. 3) as compared to control-Ab treated animals or HX no treatment (Arvanian et al., 2009). The amplitude of responses from L1 was significantly higher as well $(3.8 \pm 0.6$ mV, n = 7, $p < 0.05$, Fig. 3) compared to HX no treatment animals (Arvanian et al., 2009) or animals from control-Ab treated group (Fig. 3E). These results suggest that chronic treatment with NG2-Ab may improve transmission to L5 motoneurons through VLF fibers from rostral and caudal to injury epicenter spinal segments after HX SCI.

3c. *Neutralization of NG2 by NG2-Ab improves anatomical plasticity.*

FR Retrograde transport. In order to study whether the beneficial effects of NG2-Ab on synaptic transmission may be associated with improved anatomical plasticity, we used the retrograde axonal transport of Fluororuby (FR) to examine intra-spinal connectivity rostral and caudal to the lesion site. As in the electrophysiological experiments, we used four animal groups: (1) non-injured (n = 5), (2) HX with no-treatment (n = 5), or (3) HX and control-Ab (n = 6) and (4) HX+ NG2-Ab (n = 6).

Eight weeks after injury, FR was injected into L5 gray matter ipsilateral to HX at the level corresponding to the position of the recording electrode in electrophysiological experiments. Two weeks after FR injections, we assessed the distribution of labeled cells in lumbar (L1-L2 segments, caudal to HX) and thoracic (T4-T7 segments, rostral to HX) grey matter, at the levels corresponding to the position of stimulation electrodes in lateral white matter in electrophysiological experiments (Fig. 4).
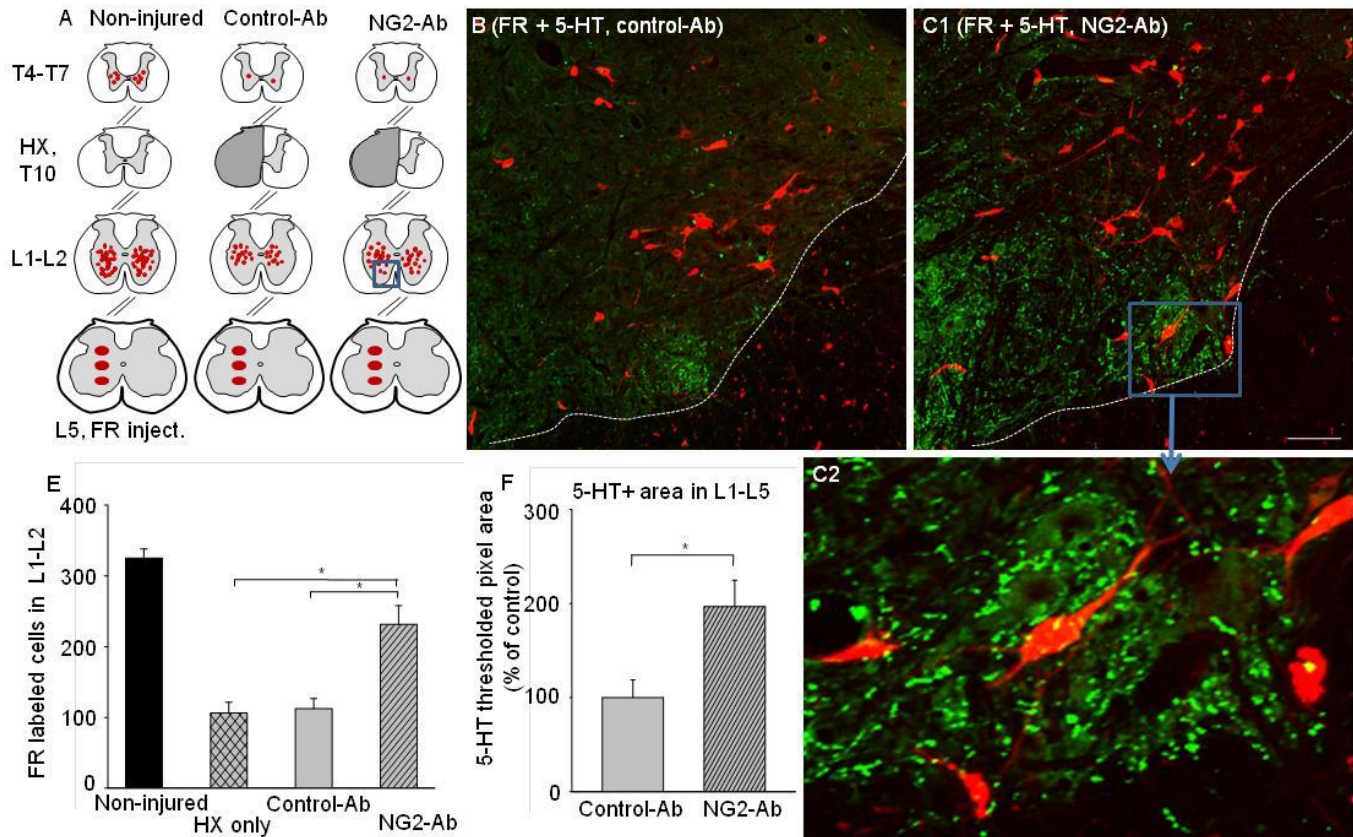

Figure 4. Chronic treatment with NG2-Ab improved retrograde transport of Fluororuby (FR) and density of 5-HT positive fibers in Lumbar segment of chronic HX animals. A, Schematic representation of FR injection site at L5 grey matter and distribution of FR-labeled cells at L1-L2 and T4-T7 spinal segments in non-injured and HX rats treated with NG2-Ab and control-Ab respectively. B, C, Representative images of lumbar sections from FR experiment stained for 5-HT-positive (5-HT+) fibers of injured animals treated with control-Ab (B) or NG2-Ab (C1), respectively. Dotted lines in B, C represent white-gray matter boundary; Scale bar = 100 µm. C2, Higher power image of boxed area in (C1) to demonstrate high density of 5-HT-positive fibers in lumbar ventral horn in the vicinity of neurons. D, Example of Cresyl Violet stained spinal cord cross section at the injury epicenter to show extend of injury with highlighted spared white matter. E, Summary of results demonstrating a significantly higher number of labeled cells in L1-L2 segment of NG2-Ab treated animals compared to control-Ab treated or HX only animals. F, Summary of results demonstrating a significantly higher 5-HT+ positive area in L1-L5 segments in NG2-Ab versus control-Ab animals. Asterisks represent significant difference between corresponding graphs (p < 0.05). Number of animals in each group after exclusion is denoted in Result section.

In non-injured animals we found many tracer-filled neurons in lumbar L1-L2 segments; fewer, but still many neurons were retrogradely traced in T4-T7 segment (Fig. 4A). These FR-labeled neurons are most probably commissural propriospinal neurons projecting from upper (T4-T7 and L1-L2) spinal segments to lower (L4-L5) segments through VLF, as previously suggested (Conta and Stelzner, 2004; Reed et al., 2006; 2009). There was a dramatic reduction in the number of labeled cells in both L1-L2 and T4-T7 spinal segments of injured animals compared to non-injured control animals (Fig. 4). The total mean number of labeled cells in L1-L2 segments was 325 ± 13 in non-injured vs 106 ± 15 in HX rats (p < 0.05). In thoracic T4-T7 level the number of FR-filled cells was 32 ± 2 in non-injured controls and only a few cells (2 ± 0.7) were retrogradely labeled in

HX rats (p < 0.05, Fig. 5). In the HX control-Ab group, the total number of FR-labeled cells at both L1-L2 (112 ± 14) and T4-T7 (2.6 ± 1.2) levels was also reduced dramatically compared to the non-injured cord and was not significantly different from the HX-only group. Importantly, the total number of FR-filled cells in L1-L2 segments of NG2-Ab treated animals was significantly higher (231 ± 26) compared to both the HX non-treated (~106 cells) and control-Ab (~112 cells) groups (Fig. 5, p < 0.05). It should be noted, however, that the number of labeled cells in T4-T7 segments in HX NG2-Ab group remained extremely low and it was not significantly different vs HX non-treated and HX control-Ab treated groups (Fig. 5). The mean measured area of the spared contralateral white matter at the injury epicenter in dorsal columns (0.19 ± 0.04 mm2 NG2-Ab group vs. 0.21 ± 0.03 mm2 control Ab group; p > 0.05) and lateral columns (1.29 ± 0.07 mm2 NG2-Ab group vs. 1.35 ± 0.06 mm2 control Ab group; p > 0.05) was not significantly different. These results suggest that neutralization of NG2 may either prevent cell death, partially preserve axonal connections between segments L1 and L5 or restore/maintain axonal transport in these nerve fibers. These anatomical results are in a good agreement with electrophysiological recordings (Fig. 3) demonstrating the enhanced synaptic plasticity in the L1-L5 segment of the injured spinal cord.

5-HT immunoreactivity. Most regenerating fibers in the injured spinal cord are serotonergic (Holmes et al., 2005; Kim et al., 2006; van den Brand et al., 2012) including those that sprout after treatment with ChABC (Barritt et al., 2006; Tom et al., 2009; Alilain et al., 2011). Therefore, we stained sections with antibody against 5-HT to measure any effects of NG2-Ab treatment on sprouting and regeneration. As shown in Figure 4 there was significantly higher 5-HT immunoreactivity in ventral areas of L1-L5 segments in rats that received HX injury and NG2-Ab treatment versus control-Ab group. These results suggest that treatment with NG2-Ab can result in enhanced density of fibers around neurons caudal to the lesion. Descending serotonergic inputs are known to be involved in locomotion function (Jordan et al., 2008), and recovery of 5-HT immunoreactivity in damaged spinal cords, particularly at lumbar levels, was shown to correlate with locomotor recovery following spinal cord injury in rats (Saruhashi et al., 1996; Kim et al., 2006; Jeong et al., 2011).

4. During Year2 we have also examined cellular and molecular mechanisms underlying effects of NG2-Ab on transmission deficits in chronically damaged spinal cord. How does NG2-Ab treatment increase transmission through VLF fibers? In order to examine possible effects of NG2-Ab on the physiological functions of the spared axons we conducted extracellular recording and intra-axonal recordings from individual axons in L1 white matter in NG2-Ab treated and control-Ab treated animals as previously described (Hunanyan et al., 2011 (note that these intra-axonal recordings were taken at position corresponding to the position of one of the stimulation electrodes described in Fig. 3). Amplitude of volley responses is known as an excellent marker of axonal conduction (Lloyd, 1949).

4a. Effects of NG2-Ab on axonal conduction. Recordings of extracellular volley responses from L1 lateral white matter in response to stimulation of VLF axons at T6 (rostral to HX) in chronic HX rats showed that the peak amplitude of the volley responses in control-Ab group was diminished significantly (0.11 ± 0.01 mV, n = 6 rats, p < 0.05) compared to previously reported measures in non-injured rats (~0.3 mV; Hunanyan et al., 2011). Consistent with NG2-Ab induced facilitation of synaptic responses (Fig. 3), chronic HX and NG2-Ab treated animals exhibited a significantly larger amplitude of the volley responses (0.19 ± 0.02 mV, n = 7 rats, p < 0.05) compared to HX/control-Ab group (Fig. 5A).
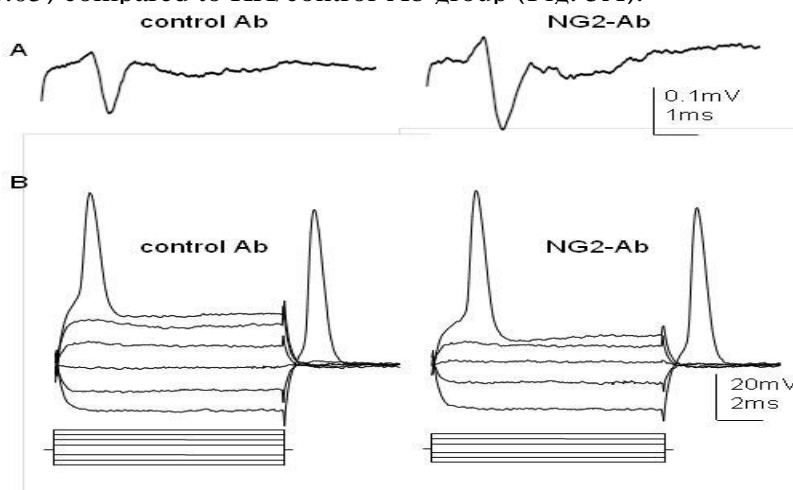

Figure 5. Intra-axonal and extra-axonal recordings from lateral white matter axons to demonstrate effect of NG2-Ab treatment on physiological properties of axons. A, Extracellular recordings of action potential volley responses to demonstrate improved conduction in NG2-Ab treated animal. Representative traces of the volley of

APs recorded extracellularly from ventrolateral funiculi at L1 segment and evoked by electric stimulation of VLF at T6 contralateral to HX in NG2-Ab and control-Ab treated chronic HX rats. B, Intra-axonal recordings from L1 VLF axons. Representative traces recorded from single axons in Control-Ab treated and NG2-Ab treated animals respectively. Both axons had a resting membrane potential of ~60 mV. Current steps (displayed below the voltage traces) of a 0.2 nA increment were applied through the recording electrode in both hyperpolarizing (to measure membrane resistance) and depolarizing directions (to trigger an AP). Note the higher rheobase but similar membrane resistance in the axon from control-Ab treated spinal cord.

These results suggest that NG2-Ab treatment may improve conduction deficits in spared axons spanning the injury epicenter after chronic HX SCI. However, this extracellular method cannot provide sufficient information whether the changes in axonal conduction are due to the effects of the neurtralizing NG2-Ab on the axonal membrane potential, input resistance or excitability.

4b. Effects of NG2-Ab on axonal excitability. In order to test any possible effect of NG2-Ab on the physiological function of the spared axons, we conducted intra-axonal recordings from individual axons in L1 white matter in NG2-Ab treated and control-Ab treated animals as described previously (Hunanyan et al., 2011). Intra-axonal recordings provide precise information about excitability of these axons (Kocsis and Waxman, 1982; Blight, 1983). These intra-axonal recordings were conducted in L1 segment; stable recordings from axons at thoracic level were not possible because immobilization of the thoracic cord interfered with breathing. For each recorded axon, we measured membrane potential and examined membrane properties applying both hyperpolarizing and depolarizing current steps through the recording electrode (Fig. 5B). By applying hyperpolarizing steps, we determined axonal input resistance and by applying depolarizing steps we determined the rheobase of axons (the minimum depolarization current required to trigger APs), a marker of axonal excitability. Reported rheobase was ~0.4 nA in non-injured and ~0.8 nA in chronically HX animals (Hunanyan et al., 2011). We found that chronic treatment with NG2-Ab significantly decreased rheobase (i.e. increased excitability) of axons compared to control-Ab treated animals (NG2-Ab: 0.5 ± 0.1 nA, n = 7 rats/34 axons vs control-Ab: 0.9 ± 0.1 nA, n = 6 rats/29 axons. $p < 0.05$, Fig. 5B). The mean input resistance of the axons acquired through hyperpolarizing current pulses was not significantly different in both groups (NG2-Ab: 79 ± 7.2 MΩ; control-Ab: 81 ± 7.6 MΩ, $p > 0.05$). The results suggest that chronic treatment with NG2-Ab partially overcomes the diminished physiological state of individual spared axons by increasing the excitability of these axons after chronic HX injury.

4c. Immunostaining for nodes of Ranvier, nodal Na-channels and NG2 in white matter contralateral to HX. The processes of NG2-expressing cells contact nodes of Ranvier (Butt et al., 1999) and exogenous NG2 accumulates along axons and at nodes of Ranvier in intact white matter (Hunanyan et al., 2010). Because nodes of Ranvier are critical for axonal excitability and the propagation of action potentials along axons (Waxman and Ritchie, 1993), we examined whether changes in excitability of spared axons revealed by electrophysiological experiments above were associated with changes in the presence of NG2 in the vicinity of Na-channels (Fig. 6).
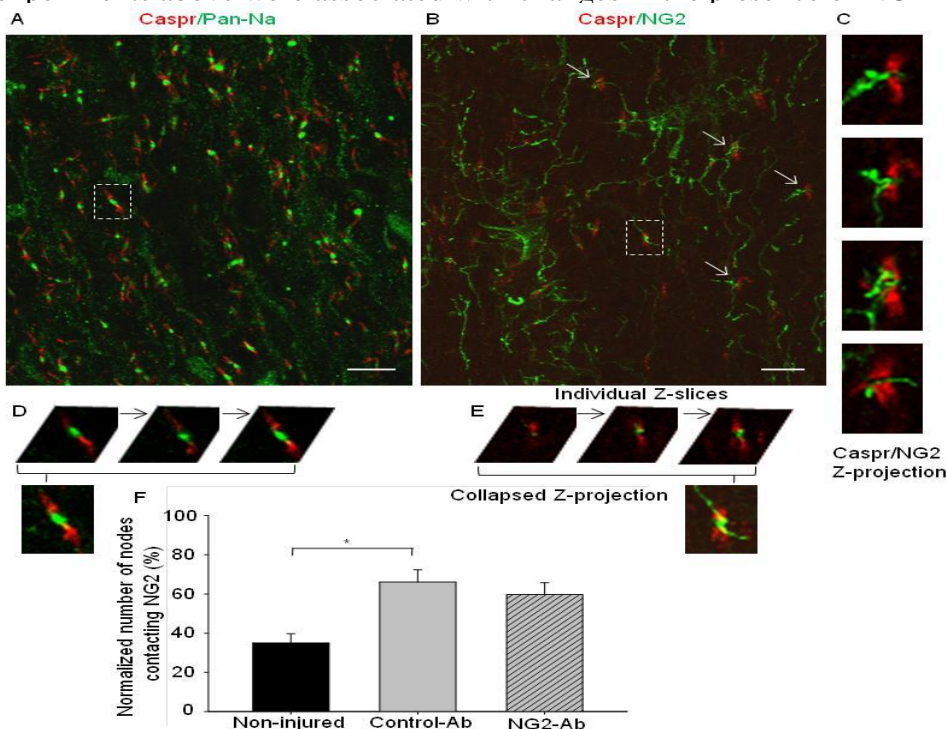
Figure 6. Confocal images of alternating longitudinal sections representing double immunostaining with Caspr/Pan-Na (A) and Caspr/NG2 (B) to show localization of Na-channels and NG2-positive processes in relation to nodes of Ranvier in the lateral white matter across to chronic HX at depth corresponding to VLF. A, Na-channel immunoreactivity was found in within almost all nodes of Ranvier. B, Many NG2-positive processes make close contacts with nodes of Ranvier. C, High power views to show NG2-positive processes in close relation to nodal gap at nodes pointed by arrows in (B). D, E, Individual Z-slices separated by 1 μm to show localization of Na-channels clusters (D) and NG2-positive processes (E) within nodal gap highlighted in A and B respectively. F, Summary of results demonstrating significantly higher number of nodes that make close contacts with NG2-positive processes within nodal gap in injured vs non-injured animals. Asterisks represent significant difference between corresponding graphs (p < 0.05). Scale bar = 10 μm.

In order to visualize the distribution of NG2-positive processes in relation to nodal Na-channels, we used double staining with Caspr/NG2 and Caspr/Na-channels (Pan-Na). Double immunostaining with Pan-Na and Caspr revealed clusters of Na-channels in virtually all nodes of Ranvier between Caspr-labeled nodal doublets (Fig. 6A). Examination of sequence of individual confocal Z-slices suggests that Na-channels cluster within the nodal gap in spared VLF axons contralateral to chronic HX white matter as they do in injured control animals (Fig. 6D). Double immunostaining with NG2 and Caspr revealed the NG2-positive processes (probably of oligodendrocyte progenitor cells) contralateral to HX white matter. Many of these NG2-positive processes appeared to be in close contact with nodes of Ranvier (Fig. 6B). The examination of individual confocal Z-slices (Fig. 6E) suggests location of NG2-positive processes within the nodal gap, corresponding to location of nodal Na-channels in alternating section. Comparison of sections double stained for Caspr and NG2 from injured and non-injured cords revealed significantly higher number of nodes with close contacts with NG2-positive processes in injured animals (Fig. 6F).

These results suggest that a possible interaction of NG2 with Na-channels within nodal gap may underlie the blocking effects of NG2 on the physiological properties of spared axons during chronic stage of HX SCI. Qualitative comparison of sections from NG2-Ab and control-Ab treated animals revealed a trend for decrease in the number of nodes contacting NG2-positive processes in NG2-Ab treated animals. However, NG2-Ab and control-Ab groups did not show statistically significant quantitative differences in the paranodal distribution of Na channels and NG2 between these 2 animal groups (Fig. 6, p = 0.47). Thus it seems that the observed increased in electrical excitability in the NG2-Ab treated cases is not due to a change in the distribution of NG2+ process at nodes of Ranvier. We cannot rule out change in nodal ultrastructure however and further electronmicroscopy studies must be conducted to understand possible mechanisms underlying effects of NG2-Ab on axonal conduction.

We hypothesize that NG2-Ab may bind to NG2 on glial cell surfaces and in the extracellular matrix and prevent an interaction of critical regions of the NG2 core protein with neurons and axonal nodes in the damaged spinal cord and thus neutralize the inhibitory function of NG2 on axonal conduction. Alternatively, binding of the antibody to NG2 stimulate internalization and/or phagocytosis of antigen-antibody complexes reducing the accumulation of NG2 at cell surfaces and in the extracellular matrix. Understanding the molecular and cellular mechanisms underlying the inhibitory function of NG2 on axonal conduction and synaptic transmission and prevention of this function by NG2-Ab requires further investigation.

5. During the past year, we have successfully created a construct for the cDNA for NG2-Ab (the Levine lab), consisting of a signal peptide, heavy chain variable region, liker region consisting of serines and glycines, light chain variable region, and a 6 histidine tag. The cDNA was then inserted into a plasmid by Integrated DNA technologies. HEK293 cells were transfected and expression of NG2-Ab has been confirmed (Fig. 7).
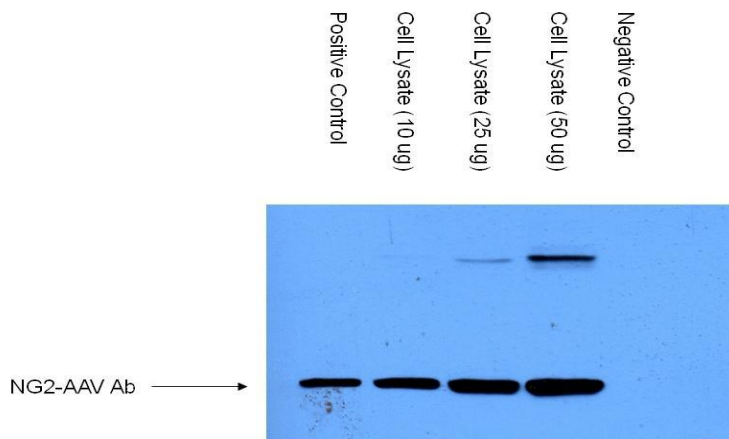

Figure 7. Western Blot analysis demonstrates that NG2-AAV Antibody is being expressed in HEK293T cells. HEK293T cells were transiently transfected with 69svIg DNA. Cell lysate was collected 48 hour post transfection for analysis. Samples were run on a 10% SDS-Page gel. Proteins were visualized with anti HIS tag antibody, and an ECL mouse IgG, HRP-linked secondary antibody

These cDNA for NG2-Ab and the plasmid that we have created, has been send to our collaborators at PENN vector core, where it is been inserted into AAV-10 viral vector. We anticipate that the construct of AAV10-NG2-Ab will be ready by the end of October of 2012. After receiving an AAV1-NG2-Ab from PENN, we will proceed with investigation of its functional effects and will examine whether prolonged delivery of NG2-Ab using this gene therapy will be a better approach to recover transmission, improve fibers growing and restore locomotor function after partial SCI.

KEY RESEARCH ACCOMPLISHMENTS:

- Anti-NG2 antibodies have been made in Levine lab and we have used these NG2-antibodies to accomplish Specific Aim 2 and portion of Specific Aims 3 and 4 related to prolonged administration of NG2-Ab via osmotic mini-pump. cDNA for NG2-Ab has been successfully created during reporting Year 2 , and portion of Specific Aims 3,4 related to examination of effects of viral vector-mediated gene expression will be conducted during final year3 of the proposal, as planned.
- We have demonstrated the ability of a single acute administration of NG2-Ab to restore conduction deficits induced by NG2 (Specific Aim 2).
- We found that prolonged delivery of anti-NG2-antibody via osmotic min-pump improved anatomical plasticity and restored transmission after chronic HX (Specific Aim 3).
- We found that delivery of anti-NG2-antibody via osmotic min-pump facilitated recovery of function after chronic SCI (Specific Aim 4).
- cDNA for NG2-Ab has been created as planned and AAV-mediated expression of NG2-Ab has been confirmed (Levine lab).
- Effects of prolonged delivery of NG2-Ab using AAV-mediated delivery of NG2-Ab expressing units on transmission and function after SCI will be accomplished during up-coming last year of the proposal.

CONCLUSIONS.

1. We have completed experiments described in Specific Aim2 of the project. We found that acute administration of NG2-antibody (Ab) prevented block of axonal conduction induced by acute NG2;

2. We have completed experiments described in Specific Aims 3 and 4 of the Project, related to the chronic delivery of NG2-Ab via osmotic mini-pump. We found that chronic delivery of NG2-Ab via intrathecal catheter and osmotic mini-pump induce partial recovery of synaptic transmission, improved anatomical plasticity and facilitated recovery of locomotor function after SCI.

3. Although this is a great proof of principle, some recent clinical studies (Novartis clinical trials) showed that similar delivery of therapeutic agents using catheter implantation may have potential problems, such as inflammations and clogging of the tip of the catheter. Thus gene therapy using AAV viral vector-mediated delivery of NG2-Ab expressing units may have a better translational potential. During Year3 we will examine therapeutic potential of AAV-NG2-Ab after SCI.

4. During 2011-2012 period we have published four papers. Some recent results described above have been summarized in two published 2012SFN and other two abstracts and two papers recently submitted for publication.

REPORTABLE OUTCOMES and REFERENCES:

Manuscripts Published in Peer- Reviewed Magazines during 2011-2012:

1. Hunanyan AS, Petrosyan HA, Alessi V, **Arvanian VL**. Repetitive spinal electromagnetic stimulation opens a window of synaptic plasticity in damaged spinal cord: role of NMDA receptors. *J Neurophysiology 2012 Jun;107(11):3027-39.*

2. G. Garcia-Alias, Hunanyan AS, Lou, , Bowers WJ, Horner PJ, Federoff HJ, Fawcett JW, Mendell LM, **Arvanian VL**. Combined administration of NT-3, NMDA-2D subunits and Chondroitinase-ABC reduces decline of transmission in damaged spinal cord and improves recovery of function after thoracic hemisection in adult rats. J Neuroscience, 31:17788-17799, 2011.

3. Hunanyan AS, Alessi V, Patel S, Pearse DD, Matthews G, **Arvanian VL.** Alterations of action potentials and the localization of Nav1.6 sodium channels in spared axons after hemisection injury of the spinal cord in adult rats. J Neurophysiol. 105:1033-44, 2011.

4. Schnell L, Hunanyan A, Bowers W, Horner PJ, Federoff HJ, Gullo M, Schwab ME, Mendell LM, **Arvanian VL.** Combined delivery of Nogo-A antibody, Neurotrophin-3 and NMDA-2D subunits establishes a functional "detour" in the hemisected spinal cord. European Journal of Neuroscience. 34:1256-1267, 2011.
(Featured article).

2012 SFN Abstracts:
1. A. S. HUNANYAN, H. A. PETROSYAN, V. ALESSI, V. L. ARVANIAN Detour synaptic connections from the motor cortex (MC) to individual neurons in lumbar dorsal and ventral horns after mid-thoracic contusion injury. Strengthening of novel connections by Chondroitinase ABC and neurotrophin NT-3.
2. Petrosyan H.A.1,2, Hunanyan A.S.1,2, Alessi V.1,2, Singh V.1, Matar N.1, Shaikh N.1, Levine J.2, Arvanian V.L. A comparison of the transduction efficiency of neurons and glial cells in adult rat spinal cord by AAV-1, 2, 5, 9, rh10 and hu11 serotypes following mid-thoracic contusion injury.

Other 2012 abstracts
Arvanian V.L. Neutralizing of inhibitory molecules (CSPGs or Nogo-A) combined with neurotrophin NT3 secretion and NMDA-NR2D expression establishes a functional "detour" in the hemisected spinal cord a in rats. 9th World Congress on Brain Injury, Edinburgh, Scotland, 2012.
Arvanian V.L. Spinal electro-magnetic stimulation combined with Neurotrophin-3 open the window of plasticity and reinforce effect of exercise after SCI in rats. American spinal Injury association (ASIA), Denver, Colorado, 2012.